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13. ABSTRACT ***(Maximum 200 Words)***

Neurofibromatosis type 2 (NF2) is a familial cancer syndrome that features the development of nervous system tumors. The *Nf2*-encoded protein, merlin, localizes to the membrane:cytoskeleton interface raising the intriguing question of how a protein that occupies such a physical niche controls cell proliferation. To generate an animal model for NF2 and to build the foundation for delineating the molecular function of merlin, we established a *Nf2*-mutant mouse strain through genetic engineering. $Nf2^{+/-}$ mice develop a spectrum of tumors that is distinct from that of their human counterparts, including osteosarcomas and hepatocellular carcinomas, which exhibit loss of the wild-type *Nf2* allele. Embryos that are homozygous for a *Nf2* mutation fail to gastrulate, while chimeric embryos partially composed of $Nf2^{-/-}$ cells develop additional defects, including during cardiac development. Together, these observations indicate a requirement for merlin function in several different cell types in the mouse; the study of Nf2 function in these cell types formed the basis of this research proposal. The following report describes progress in studying merlin function during ES cell differentiation, in cardiomyocyte specification and in liver cells. Moreover, we have established an efficient and unique system for studying merlin and ERM function in the liver *in vivo*.

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Introduction:

Neurofibromatosis type 2 (NF2) is a familial cancer syndrome that features the development of schwannomas and meningiomas of the nervous system (Huson, 1994). The identification of the *Nf2*-encoded protein, merlin, as a member of a family of proteins that localize to the membrane:cytoskeleton interface raised the intriguing question of how a protein that occupies such a physical niche controls cell proliferation (Troffatter et al., 1993;). In order to generate an animal model for NF2 and to build the foundation for delineating the molecular function of merlin, we established a *Nf2*-mutant mouse strain through genetic engineering (McClatchey et al., 1997).

As for strains of mice carrying mutations in other tumor suppressor genes, $Nf2^{+/-}$ mice are cancer prone, but develop a spectrum of tumors that is distinct from that of their human counterparts (McClatchey et al., 1998). Instead of schwannomas, $Nf2^{+/-}$ mice develop osteosarcomas, hepatocellular carcinomas and fibrosarcomas, all of which exhibit loss of the wild-type *Nf2* allele. Embryos that are homozygous for a null *Nf2* mutation fail to gastrulate, precluding our ability to directly investigate the consequences of *Nf2* homozygous loss in Schwann cells. Furthermore, chimeric embryos partially composed of *Nf2*-deficient cells develop several additional developmental defects, including during cardiac development (A.I.M., unpublished). Taken together, these observations indicate a requirement for merlin function in several different cell types in the mouse. The availability and manipulability of both wild-type and $Nf2^{-/-}$ primary mouse cells of these types yields an attractive system for delineating the molecular function of merlin. Thus the study of Nf2 function in several different mouse cell types formed the basis of this research proposal. In particular, we proposed an investigation of merlin function in cardiomyocytes during embryonic development and ES cell differentiation, and in osteoblasts and hepatocytes, from which murine *Nf2*-deficient tumors arise. Following is an update of the progress made to date.

Body:

Nf2 function during cardiomyocyte differentiation:

Our first aim was to study the requirements for merlin function in mouse development. Although *Nf2*$^{-/-}$ embryos fail to gastrulate, the defect is an extramembryonic one, and thus is easily rescued in a chimeric setting. Chimeric embryos that are partially composed of *lacZ*$^{+}$;*Nf2*$^{-/-}$ cells develop tumor-like lesions in the myocardial wall; *Nf2*- deficiency is apparently sufficient for their formation as all *lacZ*$^{+}$ cells in the myocardium form such lesions. Cardiac lesions can be detected early during heart morphogenesis (ie E9.0), suggesting that they arise via failed differentiation and morphogenesis. Thus we began by adapting an embryonic stem (ES) cell differentiation system in order to study the ability of *Nf2*$^{-/-}$ ES cells to differentiate into cardiomyocytes *in vitro*.

First we expanded and quantitated our preliminary observation that we could not observe beating foci of cardiomyocytes in differentiating cultures of *Nf2*$^{-/-}$ ES cells. It is well established that wild-type ES cells will differentiate into visually 'beating' foci of cardiomyocytes in *in vitro* cultures. Differentiation of ES cells is commonly carried out via the tightly controlled culture of ES cells in hanging drops in the absence of both adhesion and leukemia inhibitory factor (LIF). ES cells cultured in such a way will consistently form aggregates or 'embryoid bodies' (EBs) that recapitulate many of the steps of early embryonic development (Keller, 1995). Figure 1 depicts the results of a representative experiment wherein 50 wild-type and 50 *Nf2*$^{-/-}$ embryoid bodies (EBs) were plated into individual wells and monitored over the course of 14 days for visually beating foci of cardiomyocytes. While up to 30% of the wild-type EBs contained beating foci on any given day (up to 60% of the EBs beat at at least one time point), none of the *Nf2*$^{-/-}$ EBs *ever* contained beating foci. In fact, *Nf2*$^{-/-}$ EBs have been differentiated *in vitro* for over 20 days and no beating cardiomyocytes have ever been observed.

To ensure that the failure of *Nf2*$^{-/-}$ EBs to produce beating cardiomyocytes is specific to the development of the cardiac lineage and not due to a more global defect in the differentiative capacity of *Nf2*$^{-/-}$ ES cells, we compared the gross morphological features of developing wild-type and *Nf2*$^{-/-}$ EBs (Figure 2). Both wild-type and *Nf2*$^{-/-}$ EBs cultured in suspension grow with similar kinetics and form hollow cavities approximately 10 days after the initiation of differentiation as expected (Coucouvanis E. and Martin G., 1995). Embryoid bodies of both genotypes that have been fixed, sectioned and stained with hematoxylin and eosin at day 6 display no obvious differences; EBs of both genotypes exhibit an outer endodermal layer at this time. Thus there are no obvious morphological defects in the early development of *Nf2*$^{-/-}$ EBs.

To further ascertain that *Nf2*$^{-/-}$ EBs do not exhibit a global defect in differentiation, we investigated the expression of the mesodermal marker *brachyury*, in differentiating cultures of wild-type and *Nf2*$^{-/-}$ ES cells (Figure 3a). In developing embryos *brachyury* is expressed in the primitive streak which forms at the initiation of gastrulation (Wilkinson DG et al.,1990). *Brachyury* mRNA can be detected in both wild-type and *Nf2*$^{-/-}$ EBs by RT-PCR. We also examined the expression of the transcription factor *mef2C*, which is

expressed in both the cardiac and neural crest lineages during early stages of development (Edmondson DG et al., 1994) By RT-PCR, we can detect *mef2C* expression in both wild-type and *Nf2*$^{-/-}$ EBs at such stages (Leahy et al., 1999) (Figure 3b). Moreover, teratomas derived from *Nf2*$^{-/-}$ ES cells exhibit complex patters of differentiation (see below). Together these observations suggest that *Nf2*$^{-/-}$ ES cells are capable of specialized differentiation.

We have subsequently focused on the cardiac lineage in order to begin to define the defect that we see *in vivo*. First we examined the expression of fetal myosin in wild-type and
Nf2$^{-/-}$ differentiating cultures by Western blotting using the MF20 antibody, which detects embryonic, sarcomeric myosin in cardiac, and later skeletal muscle (Maltsev et al.,1993). We found that while wild-type EBs began to express fetal myosin after 10 days of differentiation, *Nf2*$^{-/-}$ EBs began to express lower levels of fetal myosin after 14 days of culture (Figure 3c). This pattern would be consistent with a lack of cardiac fetal myosin expression in *Nf2*$^{-/-}$ Ebs, but with subsequent expression in skeletal muscle (Rohwedel, et al., 1994); alternatively it is possible that cardiomyocyte differentiation is delayed in *Nf2*$^{-/-}$ EBs. Beating cardiomyocytes, however, have never been observed in *Nf2*$^{-/-}$ EBs over the course of 20 days of differentiation.

Although we have established that this *in vitro* differentiation system is appropriate for studying the consequences of Nf2 loss during cardiomyocyte development, it became apparent during the course of these studies that the hanging drop method of ES cell differentiation does not efficiently produce enough RNA or protein for these experiments to succeed. Therefore we have established a bulk culture system for ES cell differentiation; we have determined that wild-type and *Nf2*$^{-/-}$ EBs develop normally under these conditions and that we can harvest sufficient quantities of RNA and protein for multiple experiments. Thus experiments underway now aim to analyze the expression of other cardiac contractile proteins such as tropomyosin (Guan et al.,1999) or other structural features of cardiomyocytes such as connexin40, (Oyamada et al, 1996) which forms gap junctions in these cultures. Importantly, we have also generated probes for the detection by RNAse protection, of the cardiac-specific markers Nkx2.5, Gata4, Ehand and Dhand (Narita et al., 1996).

Nf2 function in teratoma formation:

A second line of experimentation aimed at investigating Nf2 function in cell differentiation has also been extended. Subcutaneous injection of ES cells in mice leads to the formation of teratomas composed of many cell lineages. We have now examined teratomas derived from three different wild-type and three different *Nf2*$^{-/-}$ ES cell lines. Although the growth rates of the two tumor types were similar, their composition was not (Figure 4). Gross anatomical comparison of the tumors reveals the striking presence of large black patches of melaocytes specifically in the *Nf2*$^{-/-}$ tumors. Moreover, the *Nf2*$^{-/-}$ tumors are clearly more vascularized than their wild-type counterparts. Histological analysis reveals that wild-type teratomas contain patches of differentiated cells of apparently endocrine, muscle, keratinocyte or neuroepithelial origin that are surrounded

by abundant fibrous connective tissue. In contrast, the *Nf2*$^{-/-}$ teratomas contained large areas of differentiated cell types with very little connective tissue. Prominent among the differentiated components of *Nf2*$^{-/-}$ tumors were large areas of primitive neuroepithelial tissues, keratinocytes and melanocytes; the increased vasculature of *Nf2*$^{-/-}$ teratomas is alsoapparent upon histological examination. We are currently examining the expression of several specific lineage markers immunohistochemically in these tumors.

Nf2 function in osteoblasts and osteosarcoma development.

Nf2$^{+/-}$ mice predominantly develop osteosarcomas of the spine and cranium; a subset of *p53*$^{+/-}$ mice also develops osteosarcomas. Moreover, we found that mice carrying compound mutations in both the *Nf2* and *p53* tumor suppressor loci exhibit markedly reduced latency of osteosarcoma development. These mice are an important model of osteosarcoma development and reflect the consequences of loss of Nf2 function in the osteoblast. We have generated a large panel of osteosarcoma cell lines derived from *Nf2*$^{+/-}$;*Nf2*$^{+/-}$;*p53*$^{+/-}$ and *p53*$^{+/-}$ mice for study. An initial characterization of the expression of markers of bone differentiation in these cell lines reveals that they all express alkaline phosphatase, bone sialoprotein and osteopontin protein by western blot analysis. As we have found these antibodies difficult to work with, we are currently confirming these observations by Northern blot analysis. Our initial data suggest that under cycling conditions in culture, tumor cell lines from these three genetic backgrounds are very similar with respect to their degree of differentiation. Experiments underway aim to examine the effect of various differentiating agents (ie vitamin D3) on the expression of these markers.

Nf2 function in hepatocytes and hepatocellular carcinoma (HCC) development.

As we have found it difficult to culture tumor cells from *Nf2*$^{-/-}$ HCCs and the incidence of these tumors in *Nf2*$^{+/-}$ mice is quite low, we have initially focused on evaluating merlin expression and regulation in liver cells. In addition, as our evaluation of HCC incidence was largely on a mixed genetic background partially composed of C57Bl/6J, which is known to be protective for chemically-induced HCC in mice, we have crossed our *Nf2* mutation onto several other genetic backgrounds that might exhibit increased HCC incidence. Preliminary evidence suggests that we have succeeded in generating *Nf2*$^{+/-}$ mice that develop HCC with higher incidence; the availability of these mice will facilitate the generation of a panel of *Nf2*$^{-/-}$ HCC cell lines for study.

Our initial work has been devoted to establishing a suitable experimental system for studying the molecular function and regulation of merlin and the related ERM proteins in mouse hepatocytes from both cultured cells and the intact liver. We previously found that merlin is regulated by phosphorylation and that merlin phosphorylation can be roughly followed by evaluating the relative levels of two major forms upon western blot analysis. The upper and lower species represent hyper- and hypophosphorylated forms of the protein, respectively. Merlin phosphorylation is regulated by cell density, growth factor availability and cell adhesion in fibroblasts; therefore we asked whether merlin is similarly regulated in liver cells. These experiments were originally proposed in primary

hepatocytes. However, since our previous observations on merlin regulation were obtained in immortalized fibroblasts, we began by utilizing an immortalized hepatoma cell line (Hepa 1-6). We favored this approach to also avoid some of the problems inherent with the use of primary cells that might interfere with the study of NF2 function such as variability among different hepatocyte preparations, adaptation to culture conditions and contamination by non-hepatocytic cells.

To test whether merlin is subjected to similar regulatory mechanisms in hepatocytes, Hepa1-6 cells were exposed to the same growth arresting (increased cell density, loss of adhesion, serum deprivation) and growth promoting (growth factor stimlation) stimuli that we previously found to control merlin phosphorylation in fibroblasts. As shown in Figure 5 (upper), only minor changes in the total or relative amount of both phosphorylated and unphosphorylated merlin were detected by western blot analysis under any of these conditions. Similarly, use of a pan-ERM antibody revealed that the banding profile of the ERM proteins was largely unaffected (Figure 5, lower). These findings suggest that in contrast to what is seen in fibroblasts, merlin is not regulated under the same conditions in these cells. Alternatively, given that these cells are derived from a benign liver tumor, it is possible that the signaling pathways that control merlin phosphorylation were deregulated during the process of tumorigenesis. Indeed, while the bulk of merlin in Hepa1-6 cells is phosphorylated and presumably inactive, the bulk of merlin isolated from normal liver tissue is unphosphorylated and presumably active (Figure 6, last two lanes). Alternatively, perhaps only a small intracellular pool of merlin is regulated under these conditions. This possibility is currently under investigation (see below).

In a complementary line of experimentation, we have also examined merlin regulation *in vivo* in the intact mouse liver. Experimental removal of 2/3 of the liver mass (partial hepatectomy; PH) in rodents causes a proliferative response in the remaining hepatocytes, restoring the original liver mass within about one week. This system has been extremely valuable in the study of the molecular events involved in both the stimulation and inhibition of liver cell proliferation since the growth response is completely arrested once the original organ size is reached (Michalopoulos et al.). We have been utilizing PH in a separate line of experimentation to try and elicit tumor formation in $Nf2^{+/-}$ mice. We therefore took advantage of the opportunity to study merlin phosphorylation in response to PH in control wild-type animals.

To investigate potential changes in merlin and the ERM proteins during the liver regenerative response, two month old 129/Sv mice were subjected to PH and sacrificed at different time intervals (from 12 hrs to 7 days). However, western blot analysis performed on total cell extracts from the excised remnant livers revealed no significant changes in ERM levels or in the levels or phosphorylation of merlin over the course of liver regeneration and return to quiescence (Figure 6). Interestingly, the ERM expression pattern from liver extracts also differs from the characteristic triplet present in Hepa1-6 cells (control lane) and cultured fibroblasts. For example, ezrin is poorly expressed in mouse liver, but not Hepa1-6 cells, indicating that the relative ERM content in hepatocytes is modified upon cell culture, immortalization or benign tumor formation.

This information will be useful when extrapolating results from *in vitro* and *in vivo* experiments.

As in the case of the above reported Hepa1-6 experiments, the absence of detectable changes in ERM levels and in merlin phosphorylation and levels in the regenerating liver suggests that merlin may not be regulated in these cells as it is in fibroblasts. Alternatively, a relatively small pool of merlin may be critically regulated. To address this possibility, we have now undertaken high resolution, gradient-based separation techniques to better define the subcellular localization of merlin isoforms and the ERM proteins.

As a complementary approach to studying merlin regulation in the liver, we would like to study the consequences of loss of Nf2 function in the liver. We originally proposed the generation of *Nf2*$^{-/-}$ hepatocytes by isolating them from chimeric embryos in years two and three of this research proposal. However, two recent events have suggested improvements on this strategy. First, other investigators have described dominant negative forms of both merlin and the ERMs. Second, a conditional allele of *Nf2* has been generated and has become available to us through Dr. Marco Giovannini (Giovannini et al).

The availability of dominant negative forms of merlin and the ERMs suggests that overexpression of these molecules *in vivo* should recapitulate loss of function mutations. Thus we have developed a suitable protocol for delivering transgene-expressing DNA vectors into the mouse liver *in vivo*. In a set of preliminary experiments we have found that tail vein injection (Zhang et al.) of a GFP-reporter, using a commercially available gene delivery system (TransIT, Mirus Corp., Madison, WI), results in the efficient expression of the GFP protein in numerous hepatocytes within the liver parenchyma (Figure 7). We have now generated several different merlin and ERM expression constructs, including both dominant negative, potentially dominant active and phosphorylation-defective alleles, which will be introduced in this manner into the mouse liver *in vivo*. Hepatocytes expressing these constructs *in vivo* will be evaluated morphologically by immunostaining for actin and cadherin, for proliferative capacity by BrdU labeling and for apoptosis by TUNEL staining. A major advantage of this approach is that the effects of expression of many constructs can be tested *in vivo* without the costly generation of multiple transgenic lines for each one.

This system can also be used in the future to introduce expression of the Cre-recombinase into *Nf2*-conditional mice. Cre-mediated recombination will mutationally inactivate *Nf2* in the cells in which it is expressed. In this way we can inactivate Nf2 specifically in adult hepatocytes *in vivo*.

Key research accomplishments:

- *In vitro* differentiation of ES cells is an appropriate system for studying the consequences of Nf2 loss during cardiomyocyte development.

- Nf2-deficient teratomas are composed of a unique spectrum of differentiated cell types. Prominent among the lineages represented in these tumors is the melanocyte. In addition, $Nf2^{-/-}$ teratomas are highly vascularized.

- Merlin phosphorylation or levels are not obviously regulated in Hepa1-6 cells in culture or during normal liver regeneration *in vivo*.

- Gene transfer to the liver can be achieved by the introduction of DNA into the tail vein of mice. Thus the effects of various dominant acting forms of merlin or the ERMs on hepatocytes can be tested *in vivo*.

Reportable outcomes:

Abstracts pertaining to the research described in this progress report have been presented at several local scientific gatherings. In addition, some of this data has been incorporated into seminar presentations given by Dr. McClatchey at several national and international meetings over the past year.

Conclusions:

We have made considerable progress in our efforts to establish systems for studying the function of the Nf2 tumor suppressor, merlin during murine ES cell differentiation. Moreover, our data suggest that this system will be amenable to delineating the defect(s) caused by loss of merlin function during cardiac morphogenesis *in vivo*. Our results thus far indicate that *Nf2*$^{-/-}$ ES cells specifically fail in their commitment to the cardiac lineage. Pinpointing the stage at which they fail during cardiomyocyte specification is our next and immediate task.

We have determined that merlin phosphorylation is not grossly regulated in Hepa1-6 hepatoma cells as it is in immortalized fibroblasts. This may indicate that a small pool of merlin is critically regulated; experiments are underway to test this hypothesis. Alternatively, the fact that these cells are derived from a mouse tumor suggests that the pathways that control merlin phosphorylation, and therefore function, may be deregulated during the process of culturing these cells or during tumorigenesis.

We have established an efficient and relatively inexpensive way of examining the effects of overexpression of various forms of merlin or the ERM proteins *in vivo* through gene transfer. These experiments will set the stage for examining the consequences of loss of merlin function in the adult liver in the near future.

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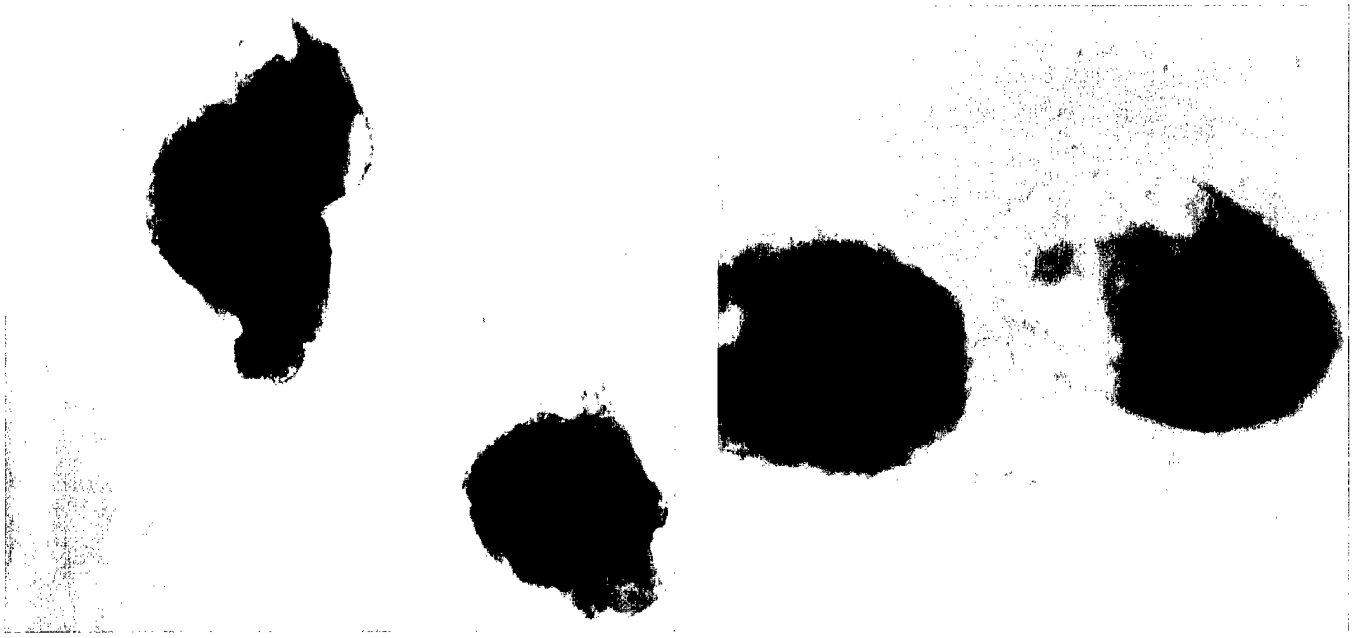
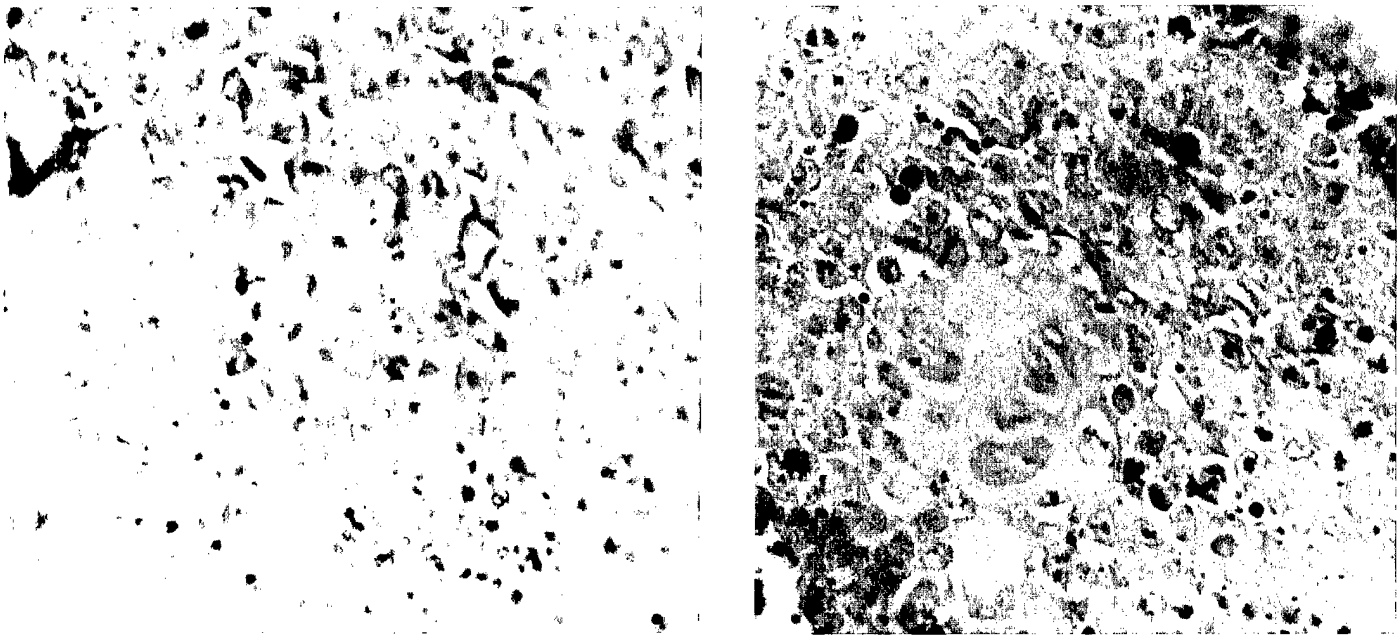


Wild-type ***Nf2*$^{-/-}$**



Wild-type ***Nf2-/-***

Figure 1. Views of embryoid bodies (EBs) cultured in suspension as well as hematoxylin and eosin stained sections of wild-type and *Nf2*$^{-/-}$ EBs. Both wild-type and *Nf2*$^{-/-}$ EBs appear morphologically similar.

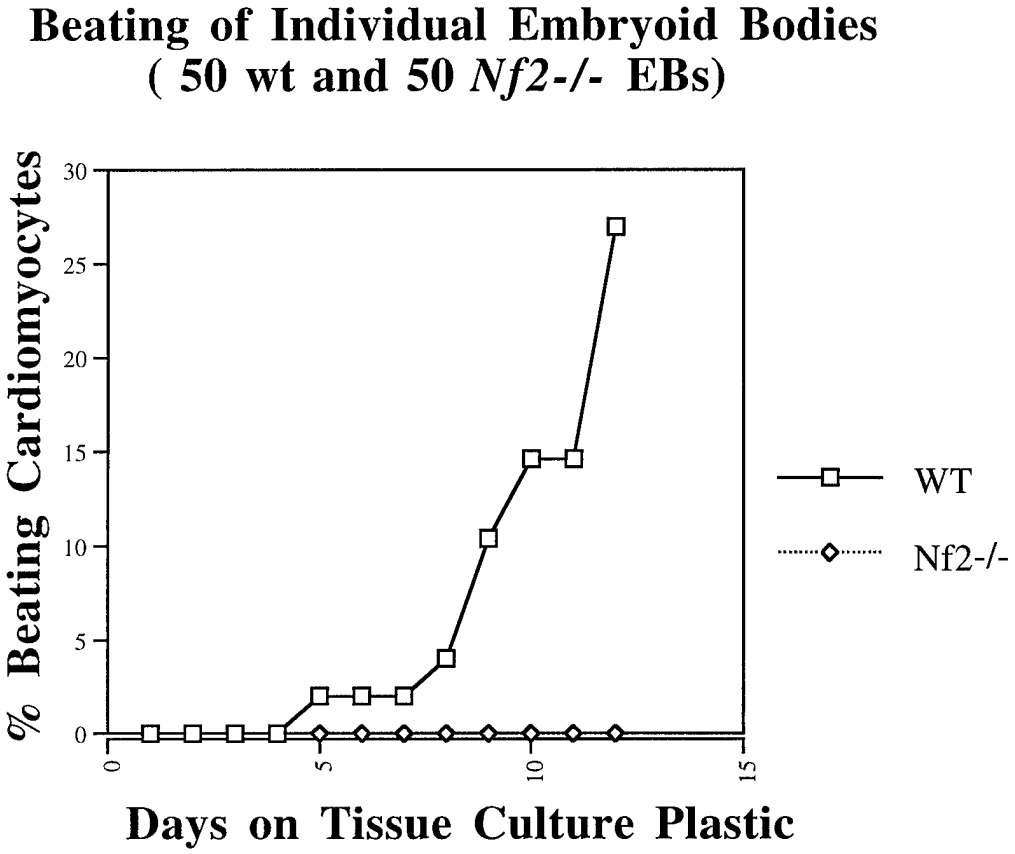


Figure 2. A representative *in vitro* differentiation experiment monitoring wild-type and *Nf2-/-* EBs. *Nf2-/-* EBs never develop beating cardiomyocytes, even when monitored for over 20 days.

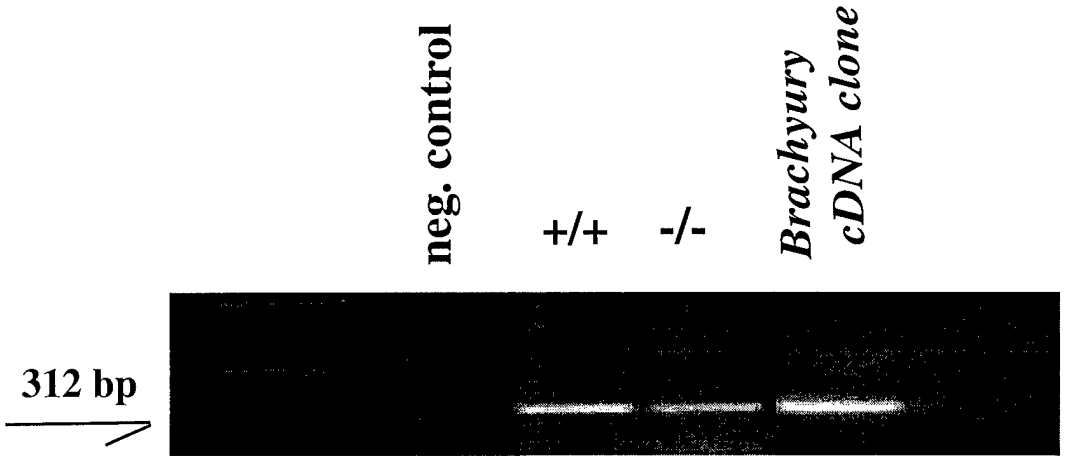


Figure 3A. RT PCR analysis of mRNA from wild-type and *Nf2*$^{-/-}$ embryoid bodies (EBs) shows that *brachyury* is expressed by both cell types. *Brachyury* is used as a marker for early mesoderm.

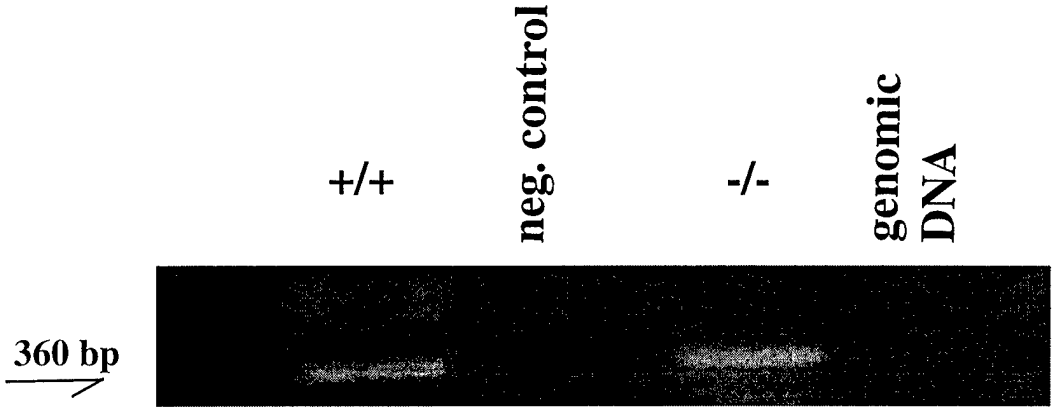


Figure 3B. RT PCR analysis shows that both wild-type and *Nf2*$^{-/-}$ EBs express *mef2C* mRNA. *Mef2C* is expressed in the developing cardiac and neural crest lineages.

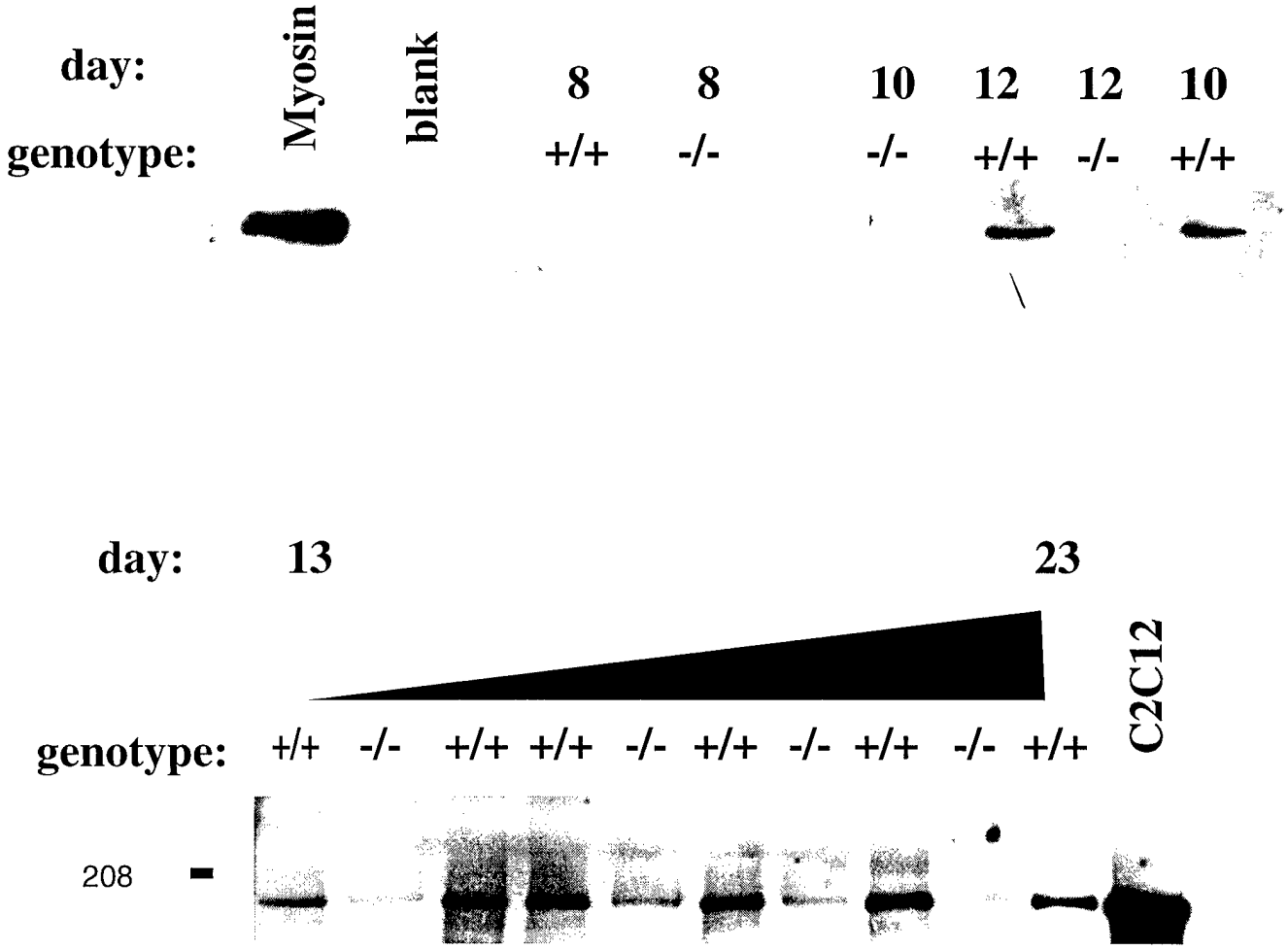


Figure 3C: The onset of myosin heavy chain (MHC) protein expression is delayed in *Nf2*$^{-/-}$ embryoid bodies. Western analysis using the MF20 antibody on a time course of wild type and *Nf2*$^{-/-}$ EBs. Thirty micrograms of protein were loaded in each lane. The myosin band of the molecular weight markers and C2C12 myoblast cell extracts serve as a positive controls.

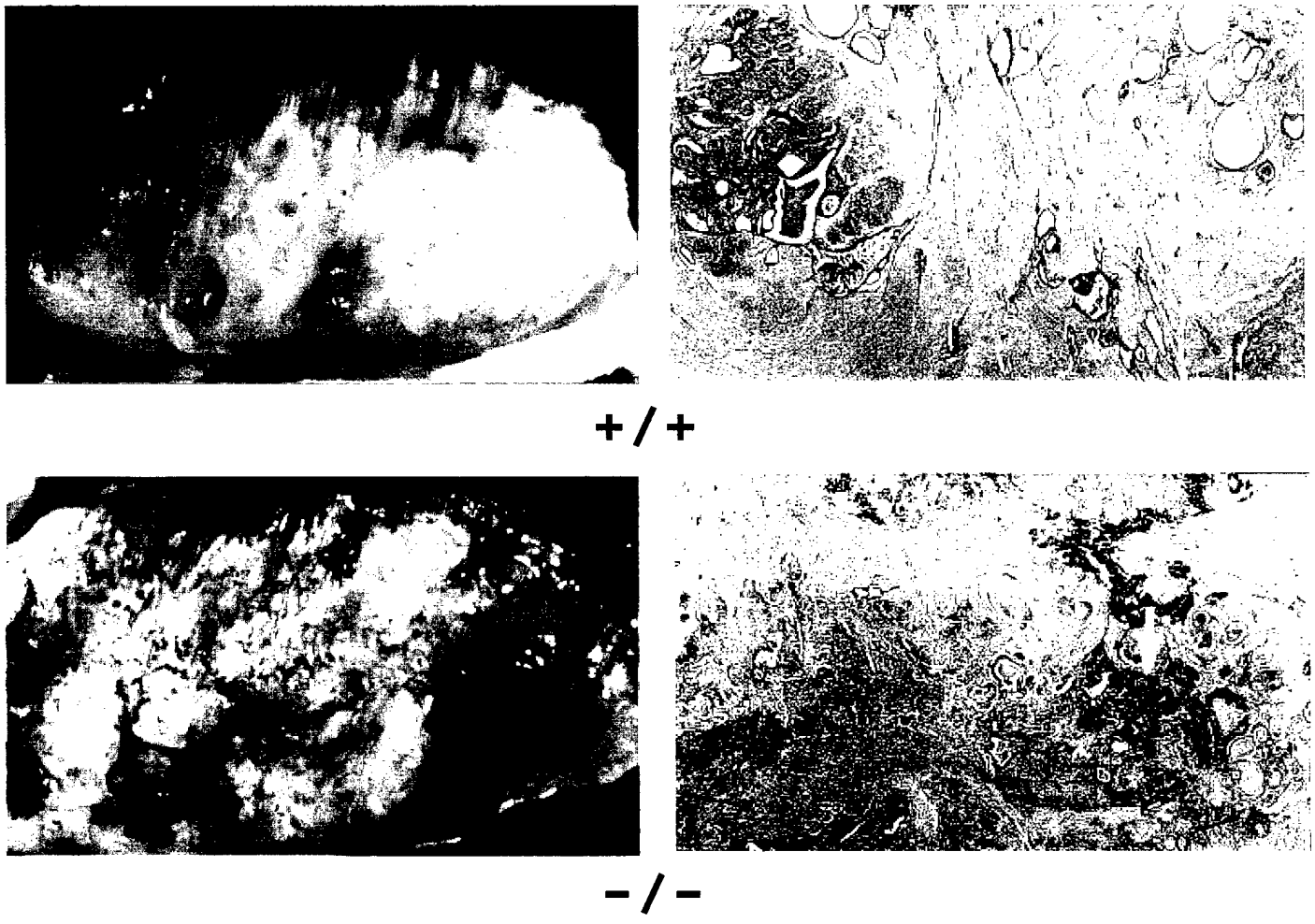


Figure 4: Teratomas derived from *Nf2*$^{-/-}$ ES cells are highly vascular and feature striking patches of melanocytes (whole mount, bottom left). Hematoxylin and eosin staining of fixed, paraffin imbedded *Nf2*$^{-/-}$ teratomas (bottom right) reveals large regions of differentiated cell types including melanocytes (arrow), keratinocytes and primitive neuroepithelium with little connective tissue. In contrast, wild-type teratomas are poorly vascularized, and are composed largely of connective tissue (top panels). Melanocytes are rarely observed in wild-type tumors.

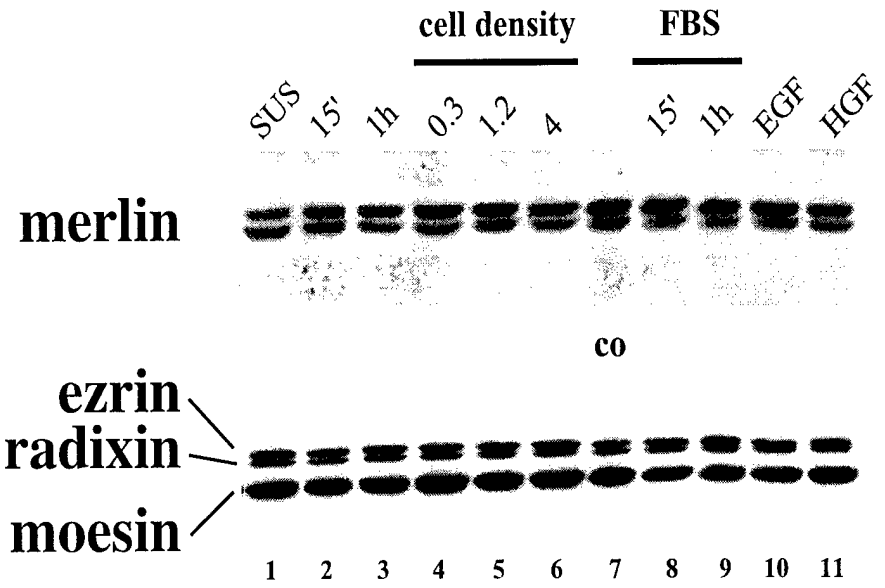


Figure 5

Immunodetection of merlin and ERM proteins in total cell extracts (50 ug/lane) from Hepa 1-6 cells subjected to different growth promoting stimuli.
Lanes 1-3: cells were maintained in suspension for 1 hour in DMEM medium containing 10% fetal bovine serum (SUS), then replated and harvested after 15 minutes (15') and one hour (1h). Lanes 4-6: cells were plated at the indicated densities (x 10^6/10 cm dishes), grown in complete medium for 24 hours, and harvested the following day. Lanes 7-11: nearly confluent cells were serum starved for 24 hours and harvested the following day before (co), and after stimulation with 20% fetal bovine serum (FBS) for 15 minutes (15') and 1 hour (1h), or 30 ng/ml Epidermal Growth Factor (EGF), or 100 ng/ml Hepatocyte Growth Factor (HGF).

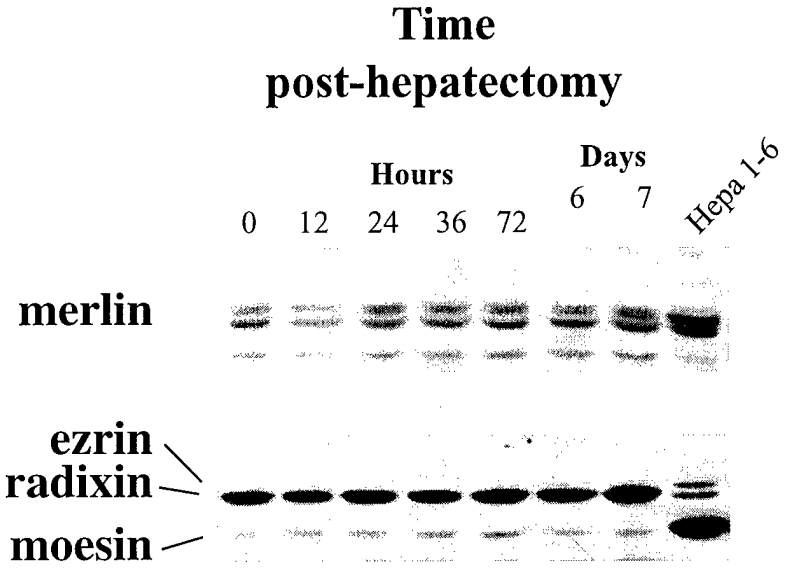
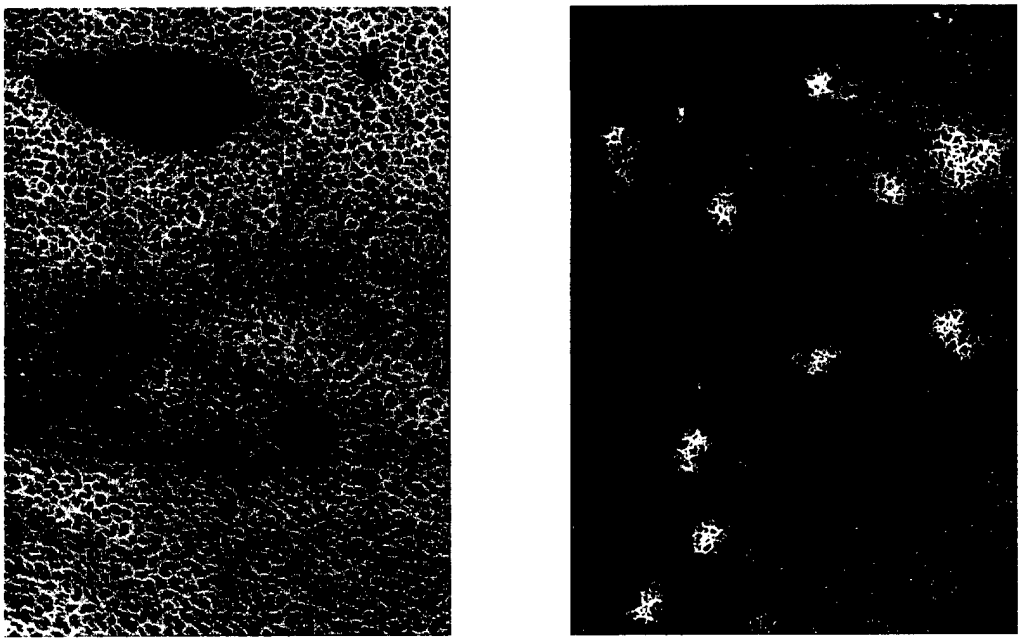


Figure 6

Immunodetection of merlin and ERM proteins in total liver extracts from partially hepatectomized 129/SV male mice. Each lane (60 ug proteins) represent pooled samples (20 ug each) of three mice per time point. The control sample is a total cell lysate from confluent, unstimulated Hepa 1-6 cells.



mock GFP

Figure 7

Detection of Green Fluorescent Protein (GFP) in air dry, unprocessed 8 um thin cryosections of livers from 129/SV male mice injected via tail vein with 20 ug GFP plasmid DNA (GFP) or carrier solution alone (mock). In vivo gene transfer of DNA was performed using the "TransIT in vivo Delivery System" (from Mirus Corporation, Madison, WI) following the manufacturer's instructions.

Annual Summary for Nested-Post-doctoral Traineeship

Award Number: DAMD17-99-1-9495

Dates covered: 15 Sep 99 – 14 Sep 00

Post-Doctoral Trainee: Marcello Curto, MD, Ph.D.

Affiliation: Massachusetts General Hospital, Cancer Center.

Principal Investigator: Andrea I. McClatchey, Ph.D.

Title: Utilization of a NF2-Mutant Mouse Strain to Investigate the Cellular and Molecular Function of the NF2 Tumor Suppressor, Merlin

I have had a long standing interest in studying the molecular mechanisms that control mammalian liver cell growth, and their alterations during the process of hepatocarcinogenesis, since graduating from medical school with a research dissertation on these topics. I have joined Dr. McClatchey's laboratory because of her recent discovery that experimental inactivation of the *Nf2* gene in mice promotes the appearance, among other metastatic tumors, of hepatocellular carcinomas. The fact that the *NF2*-encoded protein, merlin, is a member of a protein family defined as membrane-cytoskeletal linkers (previously unrelated to the process of tumorigenesis), constitute a novelty that will open a new area of investigation in the field of liver cell biology and oncogenesis.

As a member of both the Harvard biomedical community and the MGH Cancer Center, I am constantly exposed to an outstanding scientific environment which, besides being intellectually stimulating, is also a source for a highly qualified training from both a theoretical and practical standpoint. Since joining the laboratory, my initial work has been devoted to establishing suitable experimental systems to study the molecular function and regulation of the *Nf2* gene product, merlin, and the related proteins of the ERM family (ezrin, radixin, moesin), in cultured mouse hepatocytes and in the intact liver. Work done so far is summarized below.

<u>Analysis of merlin regulation in cultured hepatocytes.</u>

In cultured fibroblast cell lines, merlin has been shown to be regulated (both total levels and phosphorylation) in response to various conditions associated with both growth arrest (increased cell density; loss of adhesion; serum starvation), and growth stimulation (low cell density; serum; growth factors). In order to replicate/compare these findings in the context of a liver cell, a set of experiments was originally proposed to be conducted in primary mouse hepatocytes. However, since previous observations on merlin regulation were obtained in immortalized fibroblast cell

lines, we decided to utilize the immortalized murine hepatoma cell line Hepa 1-6 as initial experimental system. We favored this approach to also avoid some of the problems inherent with the use of primary cells that might potentially interfere with the study of Nf2 function, such as variability among different batches of hepatocyte preparations, phenotypic instability, secondary to the loss of histoarchitectural organization, the gradual adaptation to culture conditions and contamination by non-hepatocytic cells.

To determine whether merlin and the structurally related ERMs, are subject to similar regulatory mechanisms in hepatocytes, Hepa 1-6 cells were exposed to the same growth arresting and growth promoting stimuli previously tested in immortalized fibrolasts. These experiments were unable to show significant alterations of the protein levels and phosphorylation in response to the various experimental conditions tested so far. These findings suggest that, in contrast to what previously observed in fibroblasts, in cell of hepatic origin, merlin and ERM proteins might play different biological roles, the nature of which remain to be determined. A likely explanation for these results is that only a small intracellular pool of these proteins might undergo specific changes (i.e., phosphorylation, subcellular localization) that are undetectable on a standard total cell lysate. I am currently testing this possibility by developing gradient-based subcellular separation methods that will allow us to obtain a detailed profile of the intracellular distribution of merlin and the ERMs, under various experimental conditions.

Analysis of merlin regulation in liver cells *in vivo*.

The mammalian liver has the peculiar characteristic of been capable to regenerate in response to tissue damaging events (mostly chemical and infectious) that cause a reduction in the number of of liver cells. For experimental purposes, this property has been largely exploited to study several biochemical and molecular events involved in both the activation as well as the inhibition of liver cell proliferation, since the growth response is completely arrested once the original organ size is reached. Experimental surgical removal of 2/3 of the liver mass (partial hepatectomy; PH) in rodents, causes the proliferative response of all remnant hepatocytes, restoring the original liver mass within about one week. Given my previous experience with the PH procedure in rats, I have been recently perfecting this tecnique to adjust to the smaller size of mice.

In a separate line of experiments, I am investigating the changes in merlin and ERMs occurring during the liver regenerative response, in the mouse liver. Two month old 129/*Sv* male mice were subjected to PH, sacrificed at different time intervals (from 12 hrs. to 7 days), and western blot analysis was performed on total cell extracts from the excised remnant livers. As in the Hepa 1-6 experiments, no significant variations in the banding pattern of merlin were detected. Similarly, using a pan-ERM monoclonal antibody, no changes in the levels of either radixin or moesin, were detectable (ezrin is poorly expressed in mouse liver and could only be observed after prolongued film exposure or loading of higher protein amount in the gels). Interestingly, the ERM banding pattern from liver extracts differs from the one present in Hepa 1-6 and cultured fibroblasts (no ezrin and high levels of radixin in the former), indicating that the relative content

of the ERM within the hepatocyte is modified upon culturing cells in vitro. This finding will be useful when extrapolating results from in vitro to in vivo experiments and vice versa.

As in the case of the above reported Hepa 1-6 experiments, the absence of detectable changes in the total and relative levels of merlin and ERM proteins in the regenerating liver suggests that in the hepatocyte, and perhaps in epithelial cells in general- posttranslational modifications (phosphorylation) and/or subcellular distribution of a small intracellular pool of molecules might occur in response to growth controlling signals. To clarify this matter I will perform gradient-based subcellular fractionations also from liver tissue.

In vivo liver gene transfer.

In the recent months, a *Nf2*-conditional transgenic mouse has been generated in the laboratory of our collaborator Dr. M. Giovannini (INSERM-Curie Institute, Paris), and it will be soon available to us. Using this mouse it is possible to investigate the effects of a tissue-specific *Nf2* deletion induced via conditional expression of a Cre recombinase. In our case, expression of an exogenous Cre-expressing vector specifically targeted to the liver, will allow us to study the consequences of merlin loss by scattered hepatocytes within the biological context of a normal wild-type tissue.

To this end, I have been trying to develop a protocol to efficiently deliver trangene-expressing DNA vectors into the mouse liver in vivo. In a set of preliminary experiments I have found that tail vein injection of a reporter DNA vector encoding GFP (green fluorescent protein) using a commercially available gene delivery system (TransIT, Mirus Corp., Madison, WI), results in the efficient expression of the GFP protein in numerous hepatocytes within the liver parenchyma. In addition, since this system can be further exploited to deliver any desired gene into the mouse liver, I am now considering using other strategies of functional interference with the merlin/ERM proteins (i.e.: expression of dominant-negative, dominant active and other mutant alleles of merlin, ERMs, and their putative regulators).